



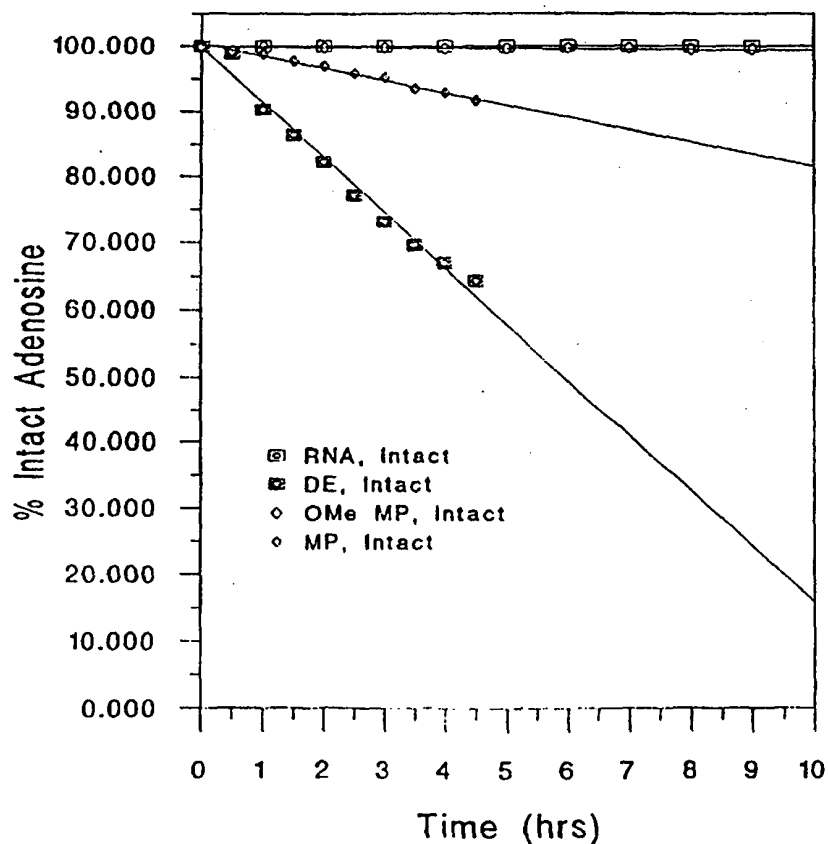
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|   |  |   |   |
|---|--|---|---|
| (51) International Patent Classification <sup>5</sup> :<br><b>A61K 31/70, C07H 21/02</b>  |  | A1  | (11) International Publication Number:<br><b>WO 94/15619</b>    |
|   |  |   | (43) International Publication Date:<br>21 July 1994 (21.07.94) |
| (21) International Application Number:<br>PCT/US94/00157  |  | (81) Designated States: AU, CA, CN, JP, KR, NZ, European patent<br>(AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC,<br>NL, PT, SE). |   |
| (22) International Filing Date:<br>5 January 1994 (05.01.94)  |  |   |   |
| (30) Priority Data:<br>08/001,109      6 January 1993 (06.01.93)      US  |  | Published<br>With international search report.  |   |
| (71) Applicants: THE JOHNS HOPKINS UNIVERSITY [US/US];<br>300 Whitehead Hall, 3400 Charles Street, Baltimore, MD<br>21218 (US). GENTA INCORPORATED [US/US]; 3550<br>General Atomics Court, San Diego, CA 92121 (US).                            |  |   |   |
| (72) Inventors: MILLER, Paul, Scott; 225 Hopkins Road, Balti-<br>more, MD 21212 (US). TS'O, Paul, On-Pong; 9039 Furrow<br>Avenue, Ellicott City, MD 21043 (US). SIMON, Lionel,<br>Norton; 15834 Highland Court, Solana Beach, CA 92075<br>(US). |  |   |   |
| (74) Agents: BIGGS, Suzanne, L. et al.; Lyon & Lyon, 34th floor,<br>611 West Sixth Street, Los Angeles, CA 90017 (US).  |  |   |   |

(54) Title: OLIGONUCLEOTIDES MODIFIED TO IMPROVE STABILITY AT ACID pH

## (57) Abstract

Acid resistant oligonucleotides modified by alkylation at the 2'-O-position are suitable for oral administration. Orally acceptable formulations prepared with the disclosed modified oligonucleotides as active ingredients are also provided.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                       |    |                          |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria                  | GB | United Kingdom                        | MR | Mauritania               |
| AU | Australia                | GE | Georgia                               | MW | Malawi                   |
| BB | Barbados                 | GN | Guinea                                | NE | Niger                    |
| BE | Belgium                  | GR | Greece                                | NL | Netherlands              |
| BF | Burkina Faso             | HU | Hungary                               | NO | Norway                   |
| BG | Bulgaria                 | IE | Ireland                               | NZ | New Zealand              |
| BJ | Benin                    | IT | Italy                                 | PL | Poland                   |
| BR | Brazil                   | JP | Japan                                 | PT | Portugal                 |
| BY | Belarus                  | KE | Kenya                                 | RO | Romania                  |
| CA | Canada                   | KG | Kyrgyzstan                            | RU | Russian Federation       |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan                    |
| CG | Congo                    | KR | Republic of Korea                     | SE | Sweden                   |
| CH | Switzerland              | KZ | Kazakhstan                            | SI | Slovenia                 |
| CI | Côte d'Ivoire            | LI | Liechtenstein                         | SK | Slovakia                 |
| CM | Cameroon                 | LK | Sri Lanka                             | SN | Senegal                  |
| CN | China                    | LU | Luxembourg                            | TD | Chad                     |
| CS | Czechoslovakia           | LV | Latvia                                | TG | Togo                     |
| CZ | Czech Republic           | MC | Monaco                                | TJ | Tajikistan               |
| DE | Germany                  | MD | Republic of Moldova                   | TT | Trinidad and Tobago      |
| DK | Denmark                  | MG | Madagascar                            | UA | Ukraine                  |
| ES | Spain                    | ML | Mali                                  | US | United States of America |
| FI | Finland                  | MN | Mongolia                              | UZ | Uzbekistan               |
| FR | France                   |    |                                       | VN | Viet Nam                 |
| GA | Gabon                    |    |                                       |    |                          |

DESCRIPTION**Oligonucleotides Modified to improve Stability at Acid pH**Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Serial No. 07/558,338, filed July 27, 1990 which is a continuation-in-part of U.S. Serial No. 06/924,234, filed  
5 October 28, 1986, the disclosures of which are incorporated herein by reference.

Background and Introduction to the Invention

The present invention is directed to methods of providing Oligomers which exhibit improved stability at  
10 acid pH, to methods of delivering such Oligomers to their sites of action and to their use in formulations for oral administration or other dosage forms where acid resistance is advantageous.

Depurination (loss of the purine bases from  
15 nucleosidyl units through cleavage of the glycosidic bond between the base and sugar) of deoxyribonucleic acid (DNA) under acidic conditions has been reported. (Hevesi, L., et al., J. Amer. Chem. Soc., 94, 4715-4720 (197)). The oral delivery of therapeutic oligodeoxyribonucleotides may  
20 require exposure of the drug to the acidic conditions of the stomach (about pH 1) for up to about 4 hours under normal conditions of drug delivery and under conditions of sustained released drug delivery (see, e.g., U.S. Patent No. 4,839,177), for up to about 12 hours. Due to its lack  
25 of stability under acid conditions, it is unlikely that enough of an orally administered oligodeoxynucleotide would remain intact to be effective. Ribonucleic acid (RNA) has been reported to be significantly more stable to depurination under acidic conditions than its DNA counter-  
30 part reportedly because of the apparent stabilizing effect

of the 2' hydroxyl on the glycosidic bond between sugar and the base (Hevesi, L., et al., supra).

Although RNA may be resistant to depurination under acid conditions, its sensitivity to ubiquitous nucleases present in biological materials limits its therapeutic usefulness. Furthermore, the use of oligoribonucleotides as a drug in its unmodified form is not feasible because of the inherent instability of the molecule to neutral to mildly basic conditions.

Unfortunately, protection of the RNA against nucleases by replacing the phosphate linkages with methylphosphonates is not possible because the 2'-hydroxyl of the sugar rapidly cleaves the methylphosphonate backbone.

#### 15 Summary of the Invention

The present invention is directed to methods of providing Oligomers which comprise nucleosidyl units having a preselected base sequence in an acid resistant form. According to one aspect of the present invention

Oligomers are provided wherein the nucleosidyl units have a sugar moiety which is a 2'-O-alkyl ribosyl group. Preferably the Oligomer is substantially neutral. Preferred are Oligomers having methylphosphonate internucleosidyl linkages more preferably from about 50 percent to about 100 percent of the internucleosidyl linkages are methylphosphonate linkages. Preferred 2'-O-alkyl ribosyl groups include 2'-O-methyl ribosyl groups.

According to an alternate aspect, the Oligomers provided in acid resistant form comprise methylphosphonate internucleosidyl linkages, preferably from about 50 percent to about 100 percent methylphosphonate linkages.

According to an additional aspect, the present invention is directed to methods of preparing an Oligomer which comprises nucleosidyl units having a preselected base sequence which Oligomer is suitable for oral administration and exhibits resistance to acid

degradation. According to one aspect this method comprises synthesizing the Oligomer using nucleosidyl units having a sugar moiety which is a 2'-O-alkylribosyl group, more preferably a 2'-O-methyl ribosyl group.

- 5 Preferably the Oligomer is substantially neutral. More preferably the Oligomer is synthesized to have methylphosphonate internucleosidyl units.

Alternatively, the method comprises synthesizing the Oligomer using nucleosidyl units having methylphosphonate  
10 internucleosidyl linkages. Particularly preferred are Oligomers having from about 50 percent to about 100 percent methylphosphonate internucleosidyl units.

In a further aspect, the present invention is directed to a method of orally delivering an Oligomer to  
15 a mammal for therapeutic purposes wherein said Oligomer comprises a nucleosidyl unit having a purine base which method comprises administration of an acid resistant Oligomer. According to one embodiment of this aspect the acid resistant Oligomer comprises nucleosidyl units having  
20 a sugar moiety which comprises a 2'-O-alkyl ribosyl group, preferably a 2'-O-methylribosyl group. Preferably the Oligomer is substantially neutral. Preferred acid resistant Oligomers include Oligomers having methylphosphonate internucleosidyl linkages, more preferably from about 50  
25 percent to about 100 percent of the internucleosidyl linkages are methylphosphonate linkages. According to an especially preferred aspect, the acid resistant Oligomer is administered in a controlled-rate release form.

Among other factors, the present invention is based  
30 on our surprising finding that Oligomers which are synthesized to incorporated nucleosidyl units having a sugar moiety which is a 2'-O-alkyl ribosyl group or which incorporate methylphosphonate internucleosidyl linkages exhibit advantageous resistance to acid catalyzed  
35 depurination and subsequent hydrolysis.

It is also believed that the Oligomers which comprise nucleosidyl units having a 2'-O-alkylribosyl group and

methylphosphonate internucleosidyl linkages appear to form more stable duplexes with an RNA target molecule than do the corresponding 2'-deoxy-ribonucleoside methylphosphonates.

5 According to a further aspect, the present invention is directed to pharmaceutical compositions which comprise an acid resistant Oligomer of the present invention in a controlled-rate release form. Alternatively, pharmaceutical compositions comprising an acid resistant Oligomer  
10 are provided which compositions are acidic themselves or which may be exposed to acidic conditions during manufacture or storage.

#### Definitions

As used herein, the following terms have the  
15 following meanings unless expressly stated to the contrary.

The term "purine" or "purine base" includes not only the naturally occurring adenine and guanine bases, but also modifications of those bases such as bases substituted at the 8-position, or guanine analogs modified at  
20 the 6-position or the analog of adenine, 2-amino purine, as well as analogs of purines having carbon replacing nitrogen at the 9-position such as the 9-deaza purine derivatives and other purine analogs.

25 The term "nucleoside" includes a nucleosidyl unit and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen-containing base. The term includes not only those nucleosidyl units having A, G, C, T and U as their  
30 bases, but also analogs and modified forms of the naturally-occurring bases, including the pyrimidine-analogs such as pseudoisocytosine and pseudouracil and other modified bases (such as 8-substituted purines). In RNA, the 5-carbon sugar is ribose; in DNA, it is a 2'-  
35 deoxyribose. The term nucleoside also includes other

analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

5

The term "phosphonate" refers to the group  $\text{O}=\text{P}-\text{R}$



10 wherein R is hydrogen or an alkyl or aryl group. Suitable alkyl or aryl groups include those which do not sterically hinder the phosphonate linkage or interact with each other. The phosphonate group may exist in either an "R" or an "S" configuration. Phosphonate groups may be used  
15 as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

The term "phosphodiester" or "diester" refers to

20

the group  $\text{O}=\text{P}-\text{O}^-$



25 wherein phosphodiester groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

A "non-nucleoside monomeric unit" refers to a monomeric unit wherein the base, the sugar and/or the  
30 phosphorus backbone has been replaced by other chemical moieties.

A "nucleoside/non-nucleoside polymer" refers to a polymer comprised of nucleoside and non-nucleoside monomeric units.

35 The term "oligonucleoside" or "Oligomer" refers to a chain of nucleosides which are linked by internucleoside linkages which is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized  
40 from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "Oligomer" refers to a

chain of oligonucleosides which have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleoside alkyl- and aryl-phosphonate analogs, alkyl- and aryl-phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleoside analogs, such as phosphotriesters and other oligonucleoside analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/nucleotide polymers wherein one or more of the phosphorus group linkages between monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, or a carbamate linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone of the non-nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

The term "alkyl- or aryl-phosphonate Oligomer" refers to Oligomers having at least one alkyl- or aryl-phosphonate internucleosidyl linkage. Suitable alkyl- or aryl-phosphonate groups include alkyl- or aryl-groups which do not sterically hinder the phosphonate linkage or interact with each other. Preferred alkyl groups include lower alkyl groups having from about 1 to about 6 carbon atoms. Suitable aryl groups have at least one ring having a conjugated pi electron system and include carbocyclic aryl and heterocyclic aryl groups, which may be optionally



substituted and preferably having up to about 10 carbon atoms.

The term "methylphosphonate Oligomer" (or "MP-Oligomer") refers to Oligomers having at least one methylphosphonate internucleosidyl linkage.

The term "neutral Oligomer" refers to Oligomers which have nonionic internucleosidyl linkages between nucleoside monomers (i.e., linkages having no positive or negative ionic charge) and include, for example, Oligomers having internucleosidyl linkages such as alkyl- or aryl-phosphonate linkages, alkyl- or aryl-phosphonothioates, neutral phosphate ester linkages such as phosphotriester linkages, especially neutral ethyltriester linkages; and as sulfamate, morpholino, formacetal, thioformacetal, and carbamate linkages. Optionally, a neutral Oligomer may comprise a conjugate between an oligonucleoside or nucleoside/non-nucleoside polymer and a second molecule which comprises a conjugation partner. Such conjugation partners may comprise intercalators, alkylating agents, binding substances for cell surface receptors, lipophilic agents, nucleic acid modifying groups including photo-cross-linking agents such as psoralen and groups capable of cleaving a targeted portion of a nucleic acid, and the like. Such conjugation partners may further enhance the uptake of the Oligomer, modify the interaction of the Oligomer with the target sequence, or alter the pharmacokinetic distribution of the Oligomer. The essential requirement is that the oligonucleoside or nucleoside/non-nucleoside polymer that the Oligomer conjugate comprises be substantially neutral.

The term "substantially neutral" in referring to an Oligomer refers to those Oligomers in which at least about 80 percent of the internucleosidyl linkages between the nucleoside monomers are nonionic linkages.

The term "neutral alkyl- or aryl- phosphonate Oligomer" refers to neutral Oligomers having neutral

internucleosidyl linkages which comprise at least one alkyl- or aryl- phosphonate linkage.

The term "neutral methylphosphonate Oligomer" refers to neutral Oligomers having internucleosidyl linkages which comprise at least one methylphosphonate linkage.

The term "acid resistant" refers to Oligomers which are resistant, in comparison to deoxyribooligonucleotides, to acid-catalyzed depurination by hydrolysis of the N-glycosyl bond.

The term "triplet" or "triad" refers a hydrogen bonded complex of the bases of three nucleosides between a base (if single stranded) or bases (if double stranded) of a target sequence, a base of a Second Strand and a Third Strand (if a single stranded target sequence) or a base of a Third Strand (if a double-stranded target).

#### Brief Description of the Drawings

Figure 1 depicts a plot of the percent intact adenine versus time for Oligomers which comprise RNA, DNA, a methylphosphonate Oligomer having a 2'-O-methyl ribosylant and a methylphosphonate Oligomer.

Figure 2 depicts a plot of the log of the percent intact adenine versus time for the same Oligomers as plotted in Figure 1.

Figure 3 depicts a plot of the percent depurination versus time at 37°C for Oligomers which comprise RNA, DNA, a methylphosphonate Oligomer having a 2'-O-methylribosyl units and a metholphosphonate Oligomer.

Figure 4 depicts a plot of the log of the percent depurination versus time at 37°C for the same Oligomers as plotted in Figure 3.

Figure 5 depicts a plot of percent intact backbone versus time for a methylphosphonate Oligomer having 2'-O-methyl ribosyl units at 37°C and pH 1.

Figure 6 depicts a melting curve for hybridization of Oligomers which comprise an oligodeoxyribonucleotide (1), a methylphosphonate Oligomer having 2'-O-methylribosyl

units (2) and a methylphosphonate Oligomer having deoxyribosyl units (3) with a DNA target.

Figure 7 depicts a melting curve for hybridization of the same Oligomers as Figure 6 with an RNA target.

- 5 Figure 8 depicts examples of dimers which comprise (a) an oligo-2'-O-methyl-ribonucleoside methylphosphonate, (b) an oligodeoxyribonucleoside methylphosphonate, and (c) an oligodeoxyribonucleoside.

### Detailed Description of the Invention

#### 10 Preferred Oligomers

- The Oligomers of the present invention comprise nucleosidyl units having a sugar moiety which is an independently selected 2'-O--alkyl ribosyl group. Suitable are alkyl groups of 1 to 5 carbon atoms.  
15 Especially preferred nucleosides have a 2'-O-methyl ribosyl group.

- Oligomer Strands having the selected internucleoside linkages may be conveniently prepared according to synthetic techniques known to those skilled in the art.  
20 For example, commercial machines, reagents and protocols are available for the synthesis of Oligomers having phosphodiester and certain other phosphorus-containing internucleoside linkages. See also Gait, M.J., Oligonucleotide Synthesis: A Practical Approach (IRL Press, 1984); Cohen, Jack S., Oligodeoxynucleotides Antisense Inhibitors of Gene Expression, (CRC Press, Boca Raton, FL, 1989); and Oligonucleotides and Analogues: A Practical Approach, (F. Eckstein, ed., 1991). Preparation of Oligomers having certain non-phosphorus-containing  
25 internucleoside linkages is described in United States Patent No. 5,142,047, the disclosure of which is incorporated herein by reference.

Preferred are Oligomers that are substantially neutral.

- 35 According to an especially preferred aspect, these Oligomers have methylphosphonate internucleosidyl

linkages. More preferably all the internucleosidyl linkages are methylphosphonate linkages. Oligomers having a mixture of methylphosphonate internucleosidyl linkage and other nucleosidyl linkages may be preferable for certain therapeutic indications and are intended to be within the scope of the present invention.

Preferably the Oligomer comprise from about 4 to about 40 nucleosides, more preferably, from about 6 to 30 nucleosides. Especially preferred are Oligomer of about 8 to about 20 nucleosides.

#### Utility and Administration

The Oligomers provided herein may form a high affinity complex with a target sequence such as a nucleic acid or a protein with a high degree of selectivity. For example, derivatized Oligomers may be used to bind with and then irreversibly modify a target site in a nucleic acid by cross-linking (psoralens) or cleaving one or both strands (EDTA). By careful selection of a target site for cleavage, one of the strands may be used as a molecular scissors to specifically cleave a selected nucleic acid sequence.

The Oligomers provided herein may be derivatized to incorporate a nucleic acid reacting or modifying group which can be caused to react with a nucleic acid segment or a target sequence thereof to irreversibly modify, degrade or destroy the nucleic acid and thus irreversibly inhibit its functions.

These Oligomers may be used to inactivate or inhibit or alter expression of a particular gene or target sequence of the same in a living cell, allowing selective inactivation or inhibition or alteration of expression. The target sequence may be DNA or RNA, such as a pre-mRNA or an mRNA. mRNA target sequences include an initiation codon region, a polyadenylation region, an mRNA cap site or a splice junction. These Oligomers could also be used to permanently inactivate, turn off or destroy genes which

produced defective or undesired products or if activated caused undesirable effects.

Since the Oligomers provided herein may form duplexes or triple helix complexes or other forms of stable  
5 association with transcribed regions of nucleic acids, these complexes are useful in "antisense" or triple strand therapy. "Antisense" therapy as used herein is a generic term which includes the use of specific binding Oligomers to inactivate undesirable DNA or RNA sequences in vitro or  
10 in vivo.

Many diseases and other conditions are characterized by the presence of undesired DNA or RNA, which may be in certain instances single stranded and in other instances in double stranded. These diseases and conditions can be  
15 treated using the principles of antisense therapy as is generally understood in the art. Antisense therapy includes targeting a specific DNA or RNA target sequence through complementarity or through any other specific binding means, in the case of the present invention by  
20 formation of duplexes or triple helix complexes.

The Oligomers for use in the instant invention may be administered singly, or combinations of Oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the  
25 foregoing general mechanisms.

In therapeutic applications, the Oligomers can be formulated for a variety of modes of administration, including oral, topical or localized administration. It may be beneficial to have pharmaceutical formulations  
30 containing acid resistant Oligomers that may come in contact with acid conditions during their manufacture or when such formulations may themselves be made acidic, to some extent, in order to more compatible with the conditions prevailing at the site of application, e.g.,  
35 the acid mantle of the skin. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition.

The Oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binding, wetting agents, disintegrants, surface-active agents, erodable polymers or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, and capsules.

10       The Oligomers of the present invention are particularly suited for oral administration which may require exposure of the drug to acidic conditions in the stomach for up to about 4 hours under conventional drug delivery conditions and for up to about 12 hours when delivered in  
15 a sustained release form. For treatment of certain conditions it may be advantageous to formulate these Oligomers in a sustained release form. U.S. Patent No. 4,839,177 to Colombo et al., the disclosure of which is incorporated herein by reference, describes certain  
20 preferred controlled-rate release systems. For oral administration, the Oligomers are formulated into conventional as well as delayed release oral administration forms such as capsules, tablets, and liquids.

These Oligomers may be particularly suited for  
25 formulation in preparations for topical administration, since the skin has an acid mantle, formulations including these acid resistant Oligomers may prove advantageous. This also can be advantageous in light of the finding that these Oligomers will cross skin and mucous membranes as  
30 described in U.S. Patent Application Serial No. 07/707,879 which is incorporated by reference. Also it may be desirable to provide formulations which include acidic media.

For topical administration, the Oligomers for use in  
35 the invention are formulated into ointments, salves, eye drops, gels, or creams, as is generally known in the art.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are  
5 used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use  
10 of nasal sprays, for example, as well as formulations suitable for administration by inhalation, or suppositories.

To assist in understanding the present invention, the following examples are included which describe the results  
15 of a series of experiments. The following examples relating to this invention should not, of course, be construed in specifically limiting the invention and such variations of the invention, now known or later developed, which would within the purview of one skilled in the art  
20 are considered to fall within the scope of the present invention as hereinafter claimed.

#### Examples

##### Example 1

##### Synthesis of 2' O-Methyladenosine Synthesis Reagents

25 A. Preparation of 5'-O-Dimethoxytrityl-2'-O-methyl-3'-O-(N,N-diisopropylamino-0- $\beta$ -cyanoethylphosphine)-N-benzoyladen-  
osine

5'-O-Dimethoxytrityl-2'-O-methyl-N-benzoyladen-  
osine (0.75 g; 1.09 mmol) (Barry Associates, Inc.) was co-  
30 evaporated 3 times with anhydrous 1/1 acetonitrile/  
diisopropylethylamine. The nucleoside was then dis-  
solved in 30 ml anhydrous acetonitrile. Diisopropyl-  
ethylamine (0.570 ml; 3.27 mmol; 3 eq.) (Aldrich) was  
added at room temperature followed by chloro-N,N-  
35 diisopropylamino- $\beta$ -cyanoethoxyphosphine (0.386 ml; 1.64  
mmol; 1.5 eq.) (ABN). After one hour the reaction was

complete as determined by TLC on silica gel plates using 50/45/5 ethylacetate/hexane/triethylamine as the eluent. The solvent was evaporated, the residue dissolved in dichloromethane, washed with saturated aqueous sodium bicarbonate, and the organic layer dried with anhydrous magnesium sulfate. The crude mixture was purified on a silica gel column that was previously treated with triethylamine to neutralize the acidity of the silica. The product was eluted with 50/49/1 ethylacetate/hexane/triethylamine. The pure fractions were pooled and dried yielding 440 mg (0.45 mmoles; 41.4%) of product.

B. Preparation of 5'-O-Dimethoxytrityl-2'-O-methyl-3'-O-(N,N-diisopropylamino-methylphosphine)-N-benzoyladenosine

5'-O-Dimethoxytrityl-2'-O-methyl-N-benzoyladenosine (1.0 g; 1.45 mmoles) (Barry Associates, Inc.) was co-evaporated 3 times with anhydrous 1/1 acetonitrile/diisopropylethylamine. The nucleoside was dissolved in 20 ml anhydrous acetonitrile. Diisopropylethylamine (1.11 ml; 6.4 mmoles; 4.4 eq.) (Aldrich) was added at room temperature followed by chloro-N,N-diisopropylamino-methylphosphine (0.582 ml; 3.2 mmoles; 2.2 eq.) (JBL Scientific). After one hour the reaction was complete as determined by TLC on silica gel plates using 50/45/5 ethylacetate/hexane/triethylamine as the eluent. The solvent was evaporated, the residue dissolved in dichloromethane, washed with saturated aqueous sodium bicarbonate, and the organic layer dried with anhydrous magnesium sulfate. The crude mixture was purified on a silica gel column that was previously treated with triethylamine to neutralize the acidity of the silica. The product was eluted with 50/49/1 ethylacetate/hexane/triethylamine. The pure fractions were pooled and dried yielding 340 mg (0.41 mmoles; 28%) of product.



Example 2Preparation of a Deoxyadenosine Tetramer Having  
Phosphodiester Internucleosidyl Linkages (Compound 1)

The tetramer was synthesized and deprotected using  
5 standard phosphoramidite procedures (see, e.g., Gait,  
M.J., Oligonucleotide Synthesis A Practical Approach,  
1984 (IRL Press) on a Milligen 8750 DNA synthesizer.  
The compound was purified using reverse-phase HPLC on a  
Whatman RAC II analytical column and a gradient of  
10 acetonitrile ("ACN") in 0.1 M triethylammonium acetate  
(0-30% ACN over 40 minutes at a flow of 1 ml/minute).

Example 3Preparation of a Deoxyadenosine Methylphosphonate  
Tetramer (Compound 2) and a 2'-O-Methyl Adenosine  
15 Methylphosphonate Tetramer (Compound 4)

Compound 2 was synthesized using 5'-(dimethoxy-  
trityl) adenosine-3'-[(N,N-diisopropylamino)methyl]-  
phosphonoamite monomer. Solid-phase synthesis was  
performed on methacrylate polymer supports with a  
20 Biosearch Model 8750 DNA synthesizer according to the  
manufacturer's recommendations except for the following  
modifications: the monomer was dissolved in acetonitrile  
at a concentrations of 100 mM. DEBLOCK reagent =  
2.5% dichloroacetic acid in dichloromethane. OXIDIZER  
25 reagent = 25 g/L iodine in 0.25% water, 25% 2,6-  
lutidine, 72.5% tetrahydrofuran. CAP A = 10% acetic  
anhydride in acetonitrile. CAP B = 0.625% N,N-  
dimethylaminopyridine in pyridine. The coupling time  
was extended to 4 minutes.

30 The dimethoxytrityl group was removed from the  
oligonucleotide at the end of the synthesis.

The oligonucleotide was then cleaved from the  
support and deprotected. The support bound oligonucleo-  
tide was removed from the synthesis cartridge and placed  
35 in a glass 1 dram vial with a screw top. The support  
was treated for 30 minutes at room temperature with 1 ml

of a solution of acetonitrile/ethanol/ $\text{NH}_4\text{OH}$  (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligonucleotide  
5 was then removed from the support and the support rinsed twice with 2 ml of 1/1 acetonitrile/water, when combined with the supernatant. The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 4 ml of 6 N HCL. The neutralized solution  
10 was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 10 ml acetonitrile, 10 ml of 50% acetonitrile/100 mM triethylammonium bicarbonate, and 10 ml of 25 mM triethylammonium bicarbonate, sequentially. After the reaction solution was passed  
15 through the column it was washed with 30 ml of water. The product was then eluted with 5 ml of 1/1 acetonitrile/water.

The oligonucleotide was purified by HPLC on a reverse phase column (Whatman RAC II) using a gradient  
20 of acetonitrile in 50 mM triethylammonium acetate.

Compound 4 was synthesized, deprotected, and purified as described for Compound 2 using the 2'-O-methyl adenosine monomer of Example 1(B) with the exception that the coupling time was extended to 3  
25 minutes to allow adequate coupling of the more sterically hindered 2'-O-methyl monomer reagent. Compound 4 was synthesized on support bound deoxyadenosine.

#### Example 4

#### 30 Preparation of a Adenosine Oligoribonucleotide Tetramer (Compound 3)

The oligoribonucleotide tetramer (Compound 3) was synthesized using 5'-O-dimethoxytrityl-2'-O-tert-butyl-dimethylsilyl-3'-O-N,N-diisopropyl- $\beta$ -cyanoethylphos-  
35 phoramidite adenosine (Millipore). The synthesis was done on a 1  $\mu$ mole scale with a Milligen 8750 automated

DNA synthesizer using standard Milligen phosphoramidite procedures with the exception that the coupling times were extended to 12 minutes to allow adequate time for the more sterically hindered 2'-O-tert-butyldimethylsilyl RNA monomer to react. The syntheses were begun on control-pore glass bound 2'-O-tert-butyldimethylsilyl adenosine (Penninsula Laboratories). All other oligonucleotide synthesis reagents were as described in Milligen's standard protocols. After synthesis, the oligoribonucleotides were handled under sterile, RNases-free conditions. Water was sterilized by overnight treatment with 0.5% diethylpyrocarbonate followed by autoclaving. All glassware was baked for at least 4 hours at 300° C. The oligonucleotides were deprotected and cleaved from support by first treating the support bound oligomer with 3/1 ammonium hydroxide/ethanol for 15 hours at 55° C. The supernatant, which contained the oligonucleotide, was then decanted and evaporated to dryness. The resultant residue was then treated with 0.6 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran (which contained 5% or less water) (Aldrich) for 24 hours at room temperature. The reaction was quenched by the addition of 0.6 mL of aqueous 2 M triethylammonium acetate, pH 7. Desalting of the reaction mixture was accomplished by passing the solution through a 10DG column (Bio-Rad) using sterile water. The desalted oligonucleotide was then dried. The compound was purified on HPLC as described for Compound 1 (see Example 2).

### 30 Example 5

#### Preparation of a 2'-O-methyl Adenosine Oligotide Tetramer Having Phosphodiester Internucleosidyl Linkages

This oligonucleotide was synthesized, deprotected, and purified as described for compound 1 with the exception that the coupling time was extended to 4 minutes to allow adequate coupling of the more

sterically hindered 2'-O-methyl reagent. The compound was synthesized on support bound deoxyadenosine.

#### Example A

##### Determination of Acid Stability of Oligomers

5 The relative stability of Compounds 1 through 5 under acidic conditions that stimulate the pH of the stomach was measured. The compounds were treated with a solution of aqueous HCl, pH 1, and the rate of glycosidic bond cleavage over time determined by the  
10 appearance of the digestion product adenine on reverse-phase HPLC chromatograms.

The reverse phase HPLC analyses were performed on a Beckman System Gold HPLC Model 126 pumps, Model 168 photodiode array detector, and Model 507 autoinjector.  
15 A Whatman RAC II ODS 3 (100 x 4.6 mm) analytical column was used for the analyses. The solvent system used was a gradient of acetonitrile in 0.1 M aqueous triethylammonium acetate, pH 7. The gradient was 0 to 2% acetonitrile over 10 minutes followed by 2 to 60%  
20 acetonitrile over 10 additional minutes. The gradient then returns to 0% acetonitrile to equilibrate the column for the next injection. The flow rate was 1 ml/minute. This gradient cleanly resolved adenine from the starting reagent and various side-products of the  
25 acid digestion.

A 100 ml stock of 0.1 M HCl, pH 1.05, was prepared in sterile water (see above for sterilization protocol) and HPLC analyzed to ensure that the region that adenine elutes was clear of baseline interference. The baseline  
30 was clean in the desired region.

The rates of depurination of the tetramers were then determined by dissolving 1 O.D. Unit <sub>260</sub> with 110  $\mu$ l injected to obtain the zero hour time point. After that 10  $\mu$ l samples were automatically sampled at either 0.5  
35 hour intervals (compounds 1 and 2) or 1 hour intervals

(compounds 3 through 5). Results are set forth in Table I below.

Figure 1 depicts a plot of the percent intact adenosine versus time for the tetramers. Figure 2 is a plot of the log of the percent intact adenosine versus time. From the log plot the  $k_A$ 's and half-lives of the rates of depurination of the tetramers were calculated. Table 1 contains those figures, as well as the relative rates of depurination as compared to the phosphodiester control (compound 1).

Table I

| Compound No.           | Rate Constant (sec <sup>-1</sup> ) @ 20°C | t <sub>1/2</sub> (hrs) | Relative Rates of Depurination |
|------------------------|---|------------------------|--------------------------------|
| 1 (phosphodiester)     | 374.4                                     | 6.66                   | 1.00                           |
| 2 (methylphosphonate)  | 70.56                                     | 35.66                  | 0.188                          |
| 3 (RNA)                | Not measurable (<1)                       | Not measurable (>3000) | Not measurable (<0.001)        |
| 4 (2' OMe methylphos.) | 2.16                                      | 1155                   | 0.0057                         |

\*Corrected for 3'-terminal deoxyadenosine.

The results clearly demonstrate that 2' O-methyladenosinyl methylphosphonates are vastly more stable (175x) than either the phosphodiester deoxyadenosine or methylphosphonate deoxyadenosine analogs. The small amount of depurination evident with the 2' O-methyladenosinyl methylphosphonate is most likely due to the 3' terminal nucleoside which is a deoxyadenosinyl residue instead of 2' OMe adenosinyl, which is not available on support. To illustrate the difference in depurination rates, after 4 hours of treatment at pH 1 about 33% of the phosphodiester deoxyadenosines, only 7% of the methylphosphonate deoxyadenosines, and just 0.28% of the 2' O-methyladenosine methylphosphonates have depurinated.

A very surprising observation was the stability of the methylphosphonate deoxyadenosinyl tetramer. The stabilizing effect of the phosphonate backbone on the glycosidic bond was unexpected.

#### 5 Example B

##### Determination of Acid Stability of Oligomers at 37°C

The relative stability of Compounds 1 to 4 was determined as described in Example A using a heat block that maintained temperature at 37°C.

10 Results are set forth in Table II below.

Table II

| Compound No.              | Rate Constant (sec <sup>-1</sup> ) @ 37°C | t <sub>1/2</sub> (hrs) | Relative Rates of Depurination |
|---------------------------|---|------------------------|--------------------------------|
| 1 (phosphodiester)        | 1800                                      | 1.39                   | 1.00                           |
| 15 2 (methylphosphonate)  | 203.9                                     | 12.24                  | 0.114                          |
| 3 (RNA)                   | Not measurable (<1)                       | Not measurable (>3000) | Not measurable (<0.001)        |
| 20 4 (2' OMe methylphos.) | Not measurable (<1)                       | Not measurable (>3000) | Not measurable (<0.0001)       |

\*Corrected for 3'-terminal deoxyadenosine.

Figure 3 depicts the rate of depurination of the adenosinyl tetramers at pH 1.0 and 37°C plotted as percent depurination versus time. Figure 4 depicts the rate of depurination of adenosinyl tetramers at pH 1.0 and 37°C plotted as log percent depurination versus time. Figure 5 depicts stability of the methylphosphonate backbone to acidic conditions at pH 1.0 and 37°C for the (2'-O-methyl A)<sub>3</sub>(dA)-methylphosphonate Oligomer. The conditions were 0.3 mM Oligomer in 0.1 M HCl, pH 1.0 at 37°C. Limit of detection was 0.2%; error was ≤ 2%. Rate was determined by increase of free adenine compared with total adeninyl

bearing species, which was followed by HPLC. Data were corrected for depurination, and cleavage of 3'-terminal deoxyadenosine for the 2'-O-methyladenosinyl methylphosphonate Oligomer.

## 5 Example C

### Acid Stability of a Oligo-2'-O-Methylribonucleoside Methylphosphonate

An oligo-2'-O-methylribonucleoside methylphosphonate (A) was prepared using suitably  
 10 protected 2'-O-methylribonucleoside methylphosphonamidite synthone. In the notation indicated below, N<sup>m</sup> indicates a 2'-O-methylribonucleoside, and the underline indicates the positions of the methylphosphonate linkages. The 5'-  
 15 internucleotide bond of the Oligomer is a phosphodiester linkage. The Oligomer was deprotected by sequential treatment with hydrazine hydrate in pyridine buffered with acetic acid, followed by treatment with a solution of ethylenediamine in 95% ethanol (1:1 v/v). The  
 20 Oligomer was purified by DEAE cellulose chromatography, followed by preparative HPLC on a C-18 reversed phase column.

|    |   |          |
|----|---|----------|
|    | r- <u>AU<sup>m</sup>A<sup>m</sup>G<sup>m</sup>G<sup>m</sup>A<sup>m</sup>U<sup>m</sup>U<sup>m</sup>G<sup>m</sup>U<sup>m</sup>C</u> | <u>A</u> |
|    | d- <u>ATAGGATTGTC</u>   | <u>B</u> |
| 25 | d-ATAGGATTTGTC  | <u>C</u> |

Oligomer A was phosphorylated using polynucleotide kinase and gamma-[<sup>32</sup>P]-ATP. The phosphorylated Oligomer was then incubated with 0.1 N HCl or 1.0 N HCl at 37°C overnight (~14 hours). The Oligomer was then analyzed  
 30 by polyacrylamide gel electrophoresis under denaturing conditions. No degradation of the Oligomer was detected under these conditions. According to our experience, incubation of the deoxyribonucleoside methylphosphonate (B) with 0.1 N HCl overnight at 37°C or with 1.0 N HCl  
 35 at 37°C for several hours would result in considerable depurination at the G and A nucleosides. This

depurination would be followed by spontaneous hydrolysis of the methylphosphonate linkages resulting in production of shorter chain-length Oligomers. Past experience has shown that purine residues in  
5 oligodeoxyribonucleotides such as (C) are even more sensitive to acid-catalyzed depurination.

The results of these experiments thus suggest that purine nucleosides in an oligo-2'-O-methylribonucleoside methylphosphonate are much more resistant to acid  
10 catalyzed depurination than are the corresponding purine nucleosides in oligodeoxyribonucleoside methylphosphonates or oligodeoxyribonucleosides. The apparent resistance of oligo-2'-O-methylribonucleoside methylphosphonates to acid catalyzed depurination could  
15 have important therapeutic consequences, particularly if these Oligomers were to be administered orally.

#### Example D

##### Hybridization of a Oligo-2'-O-Methylribonucleoside Methylphosphonate to Complementary DNA and RNA Targets

20 The hybridization properties of Oligomer 2 was determined and compared to those of Oligomers 1 and 3. Hybridization to complementary single-stranded DNA target D or RNA target E was carried out in a buffer containing 10 mM HEPES (pH 7.0), 2 mM EDTA. The  
25 resulting melting curves and melting temperatures are shown in Figures 6 and 7 (note: in these figures, N<sup>m</sup> represents a 2'-O-methylribonucleoside). The oligodeoxyribonucleotide, 1, formed stable hybrids with both the DNA and RNA targets. The oligo-2'-O-  
30 methylribonucleoside methylphosphonate, 2, did not hybridize with DNA target, but did form a very stable duplex with the RNA target. This behavior is in contrast to that of the oligodeoxyribonucleoside methylphosphonate, 3, which forms a stable duplex with  
35 the DNA target, but only a very weak duplex with the RNA target. These results suggest that oligo-2'-O-



methylnucleoside methylphosphonates may form more stable hybrids with RNA than do oligodeoxyribonucleoside methylphosphonates and thus they be more effective antisense reagents when targets against cellular or viral RNAs.

Table III

|    |                                  |   | T <sub>m</sub> |
|----|----------------------------------|---|----------------|
|    | d-G A C A A A T C C T A T        |   |                |
|    | <u>(DNA TARGET)</u>              |   |                |
| 10 | C T G T T T A G G A T A-d        | 1 | 28°C           |
|    | <u>C U*G*U*U*U*A*G*G*A*U*A-r</u> | 2 | <0°C           |
|    | <u>C T G T T T A G G A T A-d</u> | 3 | 30°C           |

Buffer: 10 mM HEPES pH 7.0  
2 mM EDTA

15

Table IV

|    |                                  |   | T <sub>m</sub> |
|----|----------------------------------|---|----------------|
|    | r-G A C A A A U C C U A U        |   |                |
|    | <u>(RNA TARGET)</u>              |   |                |
|    | C T G T T T A G G A T A-d        | 1 | 18°C           |
| 20 | <u>C U*G*U*U*U*A*G*G*A*U*A-r</u> | 2 | 20°C           |
|    | <u>C T G T T T A G G A T A-d</u> | 3 | <0°C           |

Buffer: 10 mM HEPES pH 7.0  
2 mM EDTA

Claims

1. A method of providing an Oligomer comprising nucleosidyl units having a preselected base sequence in an acid resistant form wherein the nucleosidyl units of  
5 the Oligomer have a sugar moiety which is a 2'-O-alkyl ribosyl group.
2. An Oligomer according to claim 1 wherein said Oligomer is synthesized to be substantially neutral.
3. An Oligomer according to claim 2 wherein the  
10 Oligomer is synthesized to have methylphosphonate internucleosidyl linkages.
4. An Oligomer according to claim 3 wherein said alkyl is methyl.
5. An Oligomer according to claim 4 comprising  
15 from about 50 percent to about 100 percent methylphosphonate internucleosidyl linkages.
6. A method of preparing an Oligomer which comprises nucleosidyl units having a preselected base sequence which is suitable for oral administration and  
20 exhibits resistance to acid degradation which comprises synthesizing said Oligomer using nucleosidyl units having a sugar moiety which is a 2'-O-alkyl ribosyl group.
7. An Oligomer according to claim 6 which is  
25 synthesized to be substantially neutral.
8. An Oligomer according to claim 7 wherein said Oligomer is synthesized to have methylphosphonate internucleosidyl linkages.
9. An Oligomer according to claim 8 wherein said  
30 alkyl is methyl.

n Oligomer according to claim 9 comprising  
50 percent to about 100 percent  
honate internucleosidyl units.

method of providing an Oligomer comprising  
units having a preselected base sequence in  
instant form wherein the Oligomer is  
to have methylphosphonate internucleosidyl

n Oligomer according to claim 11 comprising  
50 percent to about 100 percent  
honate internucleosidyl linkages.

method of preparing an Oligomer which  
ucleosidyl units having a preselected base  
ich is suitable for oral administration and  
sistance to acid degradation which comprises  
g said Oligomer using nucleosidyl units  
ylphosphonate internucleosidyl linkages.

n Oligomer according to claim 13 comprising  
50 percent to about 100 percent  
honate internucleosidyl units.

method of orally delivering an Oligomer to a  
therapeutic purposes, said Oligomer  
a nucleosidyl unit having a purine base which  
dministration of an acid resistant Oligomer.

method according to claim 15 wherein said  
mpries nucleosidyl units having a sugar  
h comprises a 2'-O-alkyl-ribosyl group.

method according to claim 16 wherein said  
substantially neutral.

18. A method according to claim 17 wherein said alkyl is methyl.

19. A method according to claim 18 wherein said Oligomer is a methylphosphonate Oligomer.

5 20. A method according to claim 15 wherein said Oligomer is substantially neutral.

21. A method according to claim 20 wherein said Oligomer is a methylphosphonate Oligomer.

22. A method according to one of claims 15, 16, 19  
10 or 21 wherein said Oligomer is administered in a controlled-rate release form.

23. A pharmaceutical composition which comprises an acid resistant Oligomer which comprises nucleosidyl units having a sugar moiety which comprises a 2'-O-  
15 alkyl-ribosyl group in a controlled-rate release form.

24. An Oligomer according to claim 23 which comprises at least 8 nucleosidyl units.

25. A method according to claim 23 wherein said Oligomer comprises nucleosidyl units having sugar  
20 moieties which comprise a 2'-O-methyl-ribosyl group.

26. A method according to claim 25 wherein said Oligomer is substantially neutral.

27. A method according to claim 26 wherein said Oligomer is a methylphosphonate Oligomer.

25 28. A pharmaceutical composition for oral administration which comprises an acid resistant

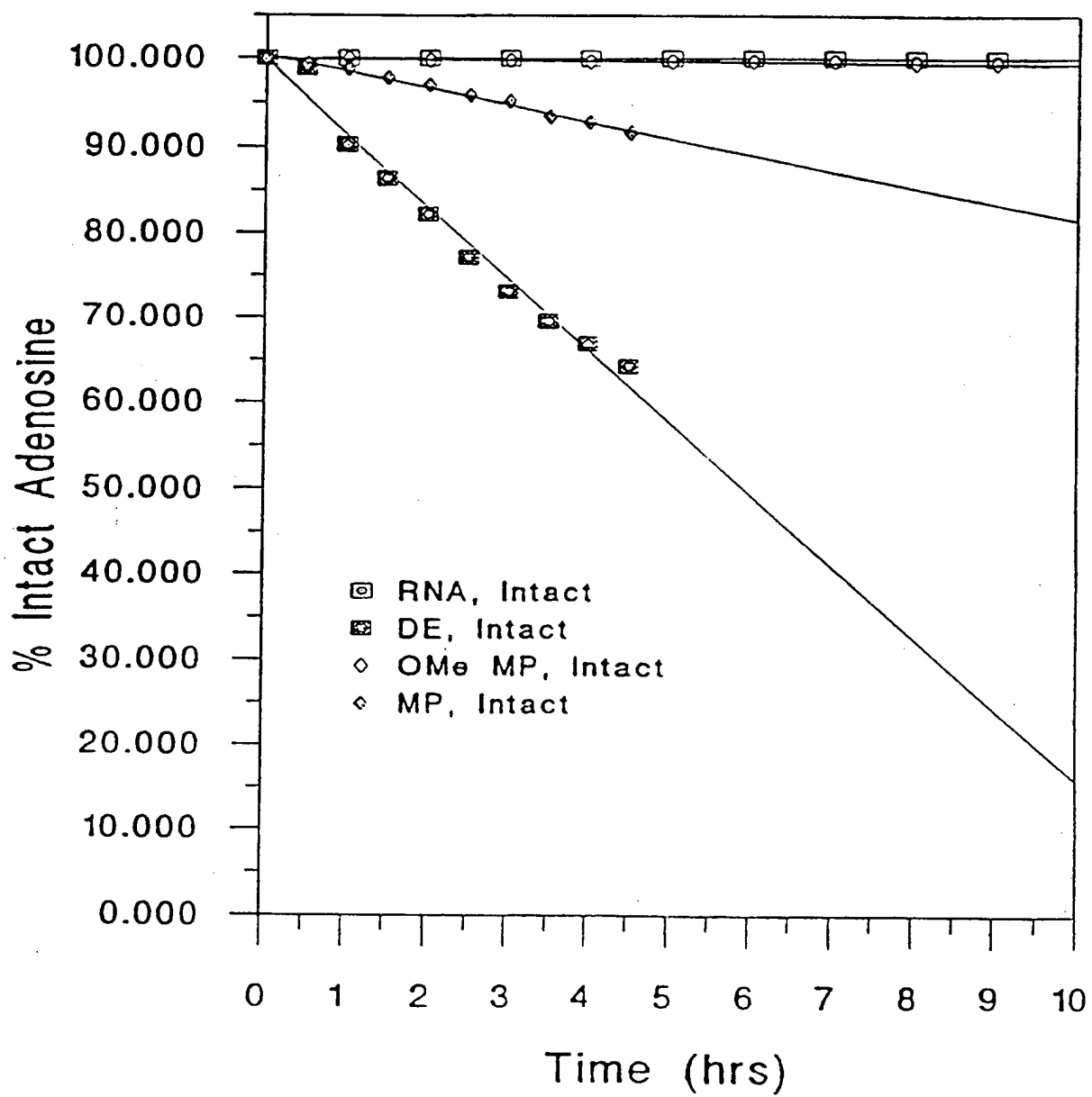
Oligomer which has methylphosphonate internucleosidyl units in a controlled-rate release form.

29. A composition according to claim 28 wherein said Oligomer comprises nucleosidyl units having a sugar  
5 moiety which is a 2'-O-methylribosyl group.

30. A pharmaceutical composition which retains its therapeutic activity under acidic conditions which comprises a therapeutically effective amount of an acid resistant Oligomer in a compatible formulation.

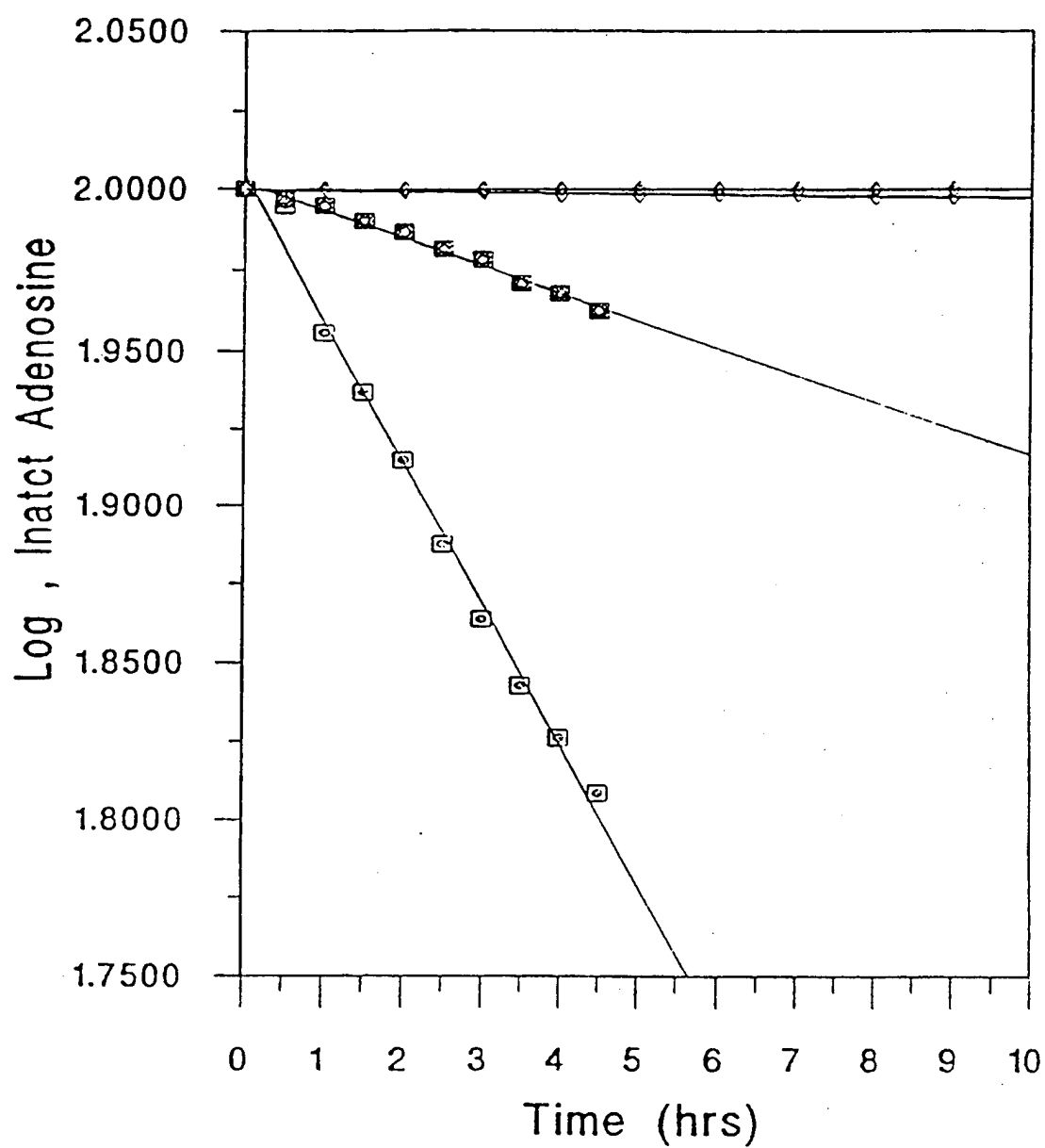
1/7

FIG. 1.



2/7

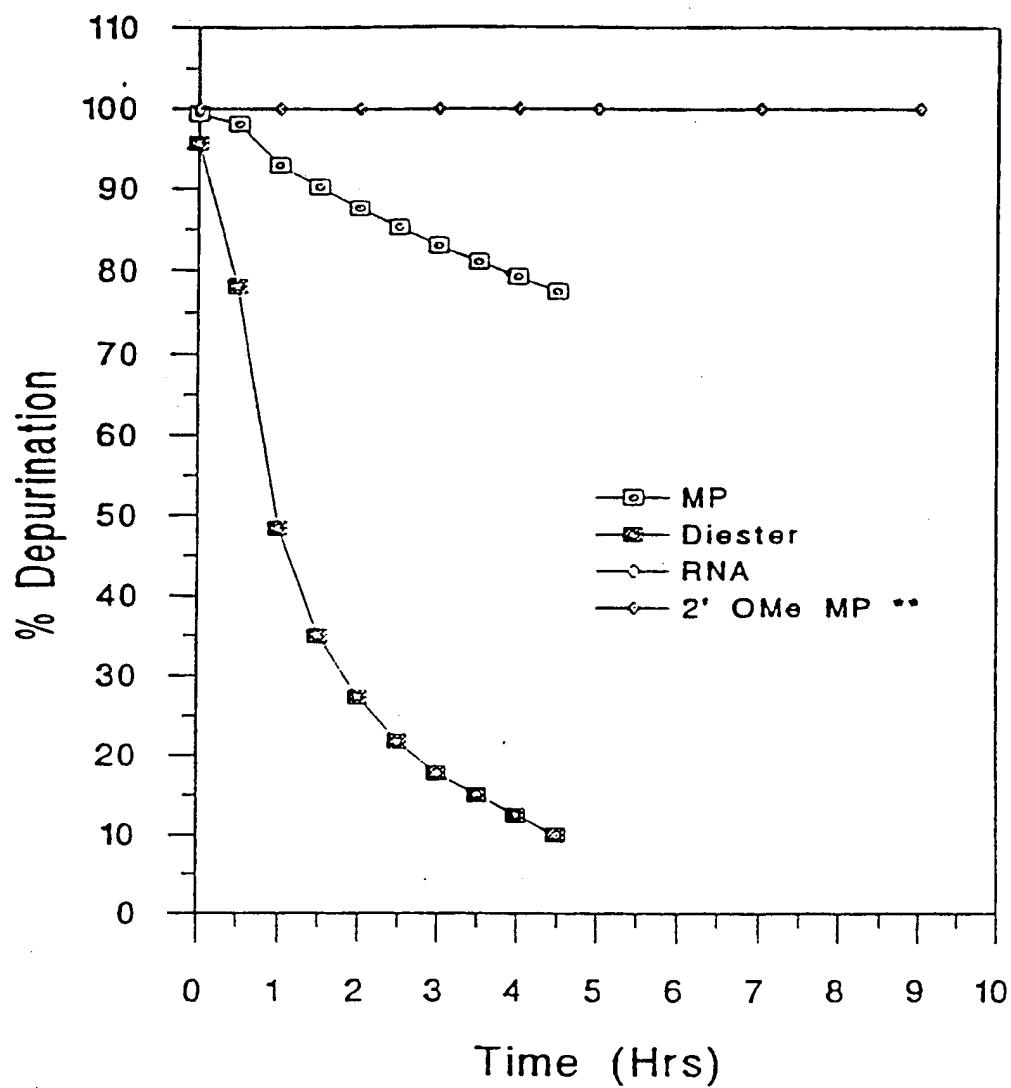
FIG. 2.



- Log, DE
- Log, MP
- ◇ Log, 2'-OMe MP
- ◆ Log, RNA

3/7

FIG. 3.

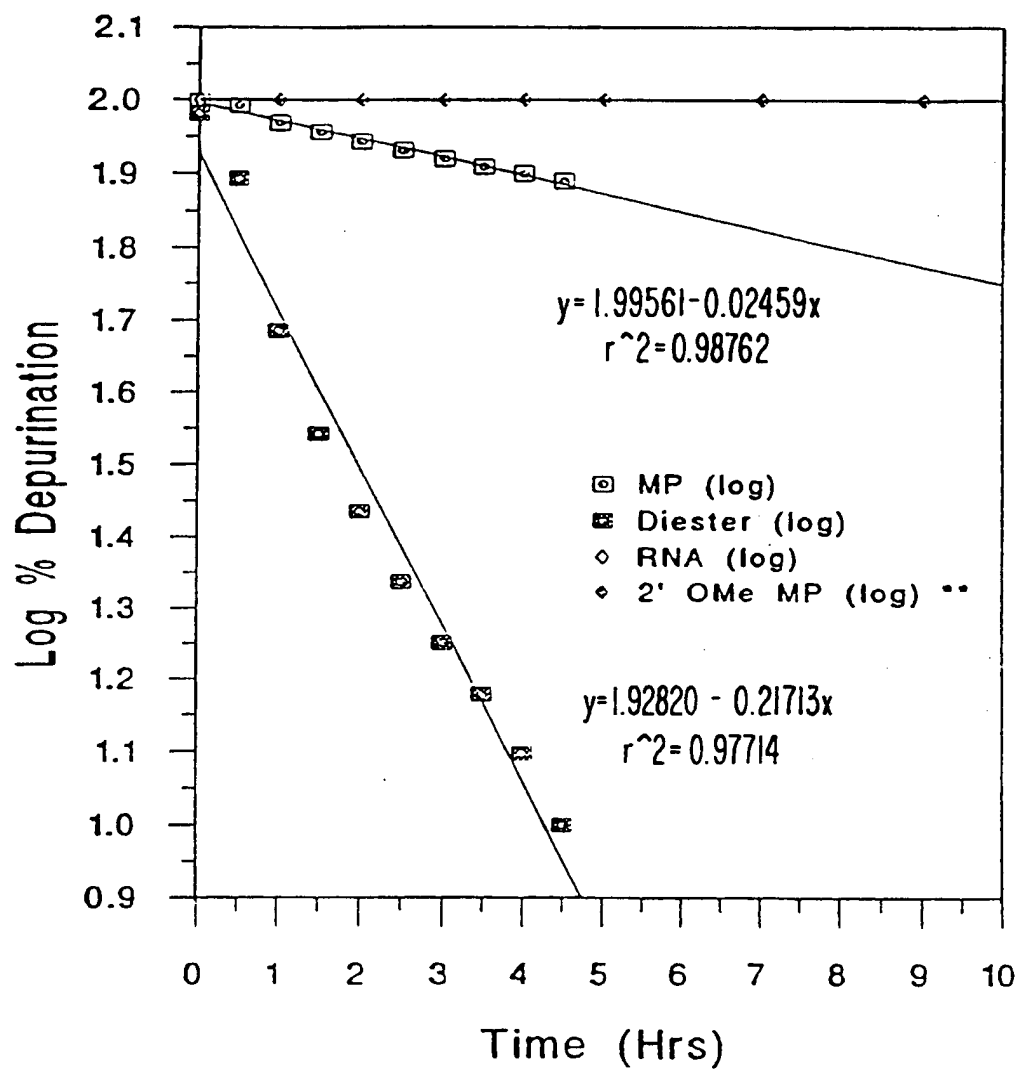


\*\* 2' OMe MP data corrected for depurination of 3' terminal deoxyadenosine.



4/7

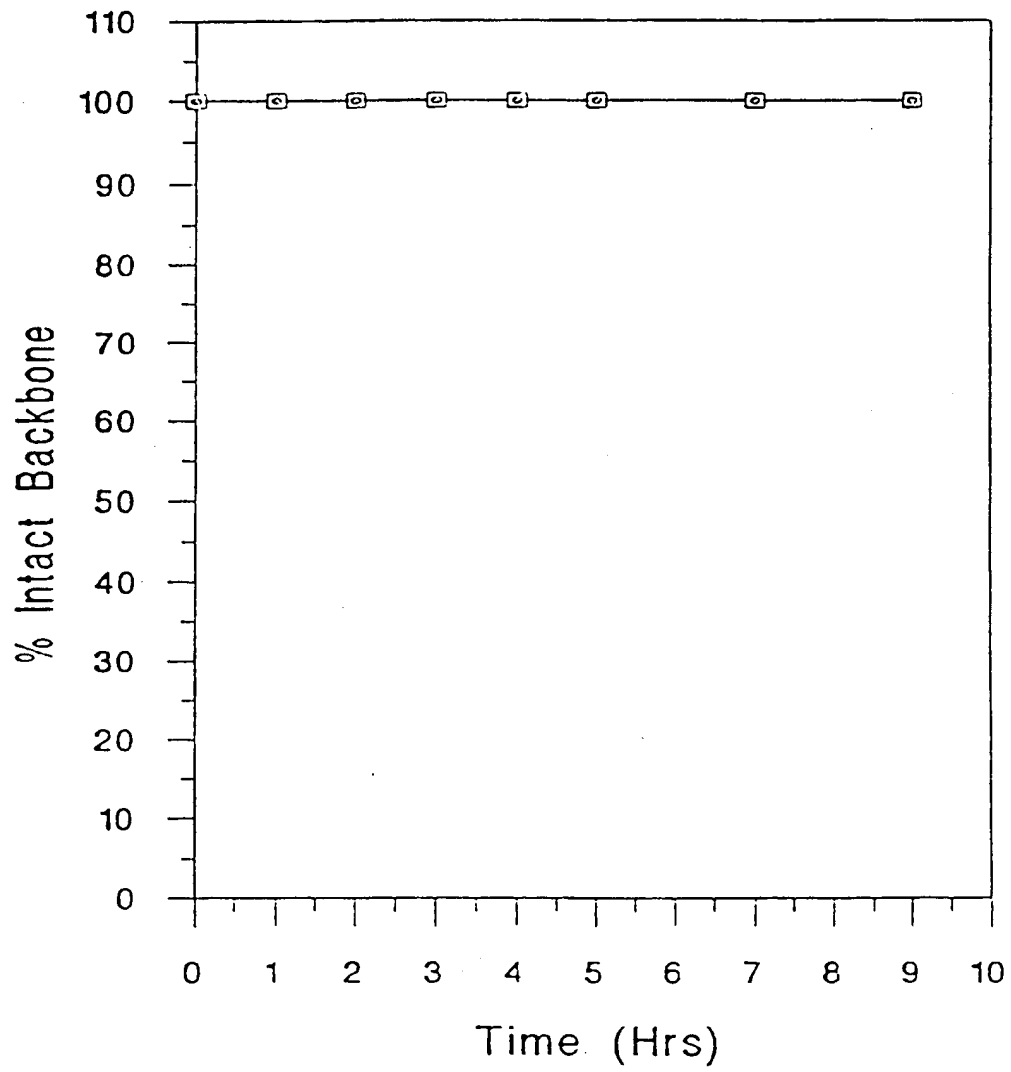
FIG. 4.



\*\* 2' OMe MP data corrected for depurination of 3' terminal deoxyadenosine.

5/7

FIG. 5.



6/7

FIG. 6.

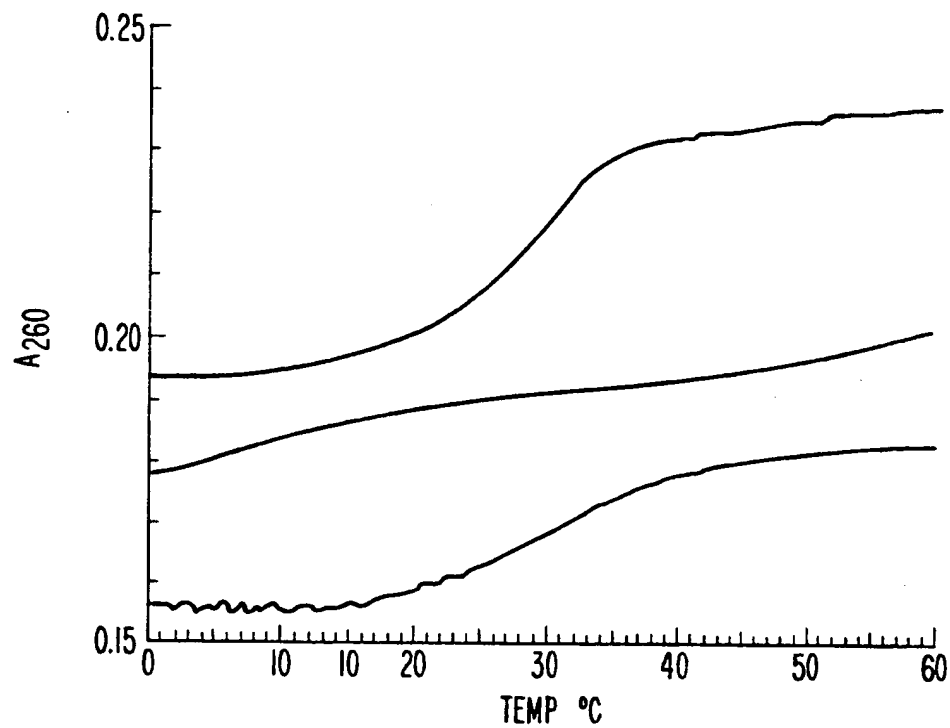
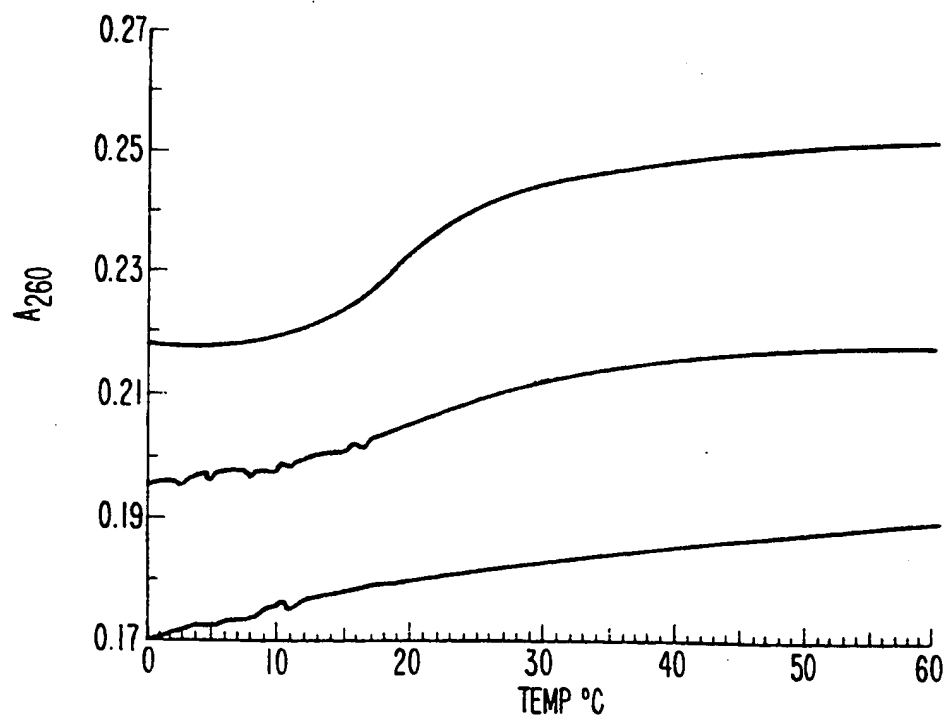
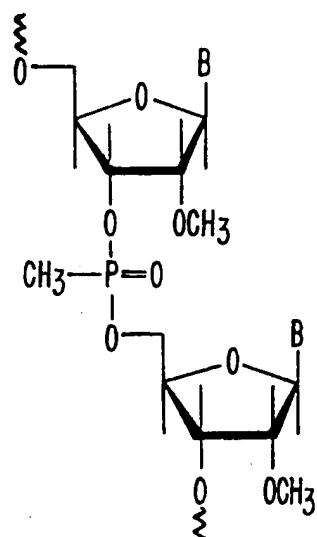


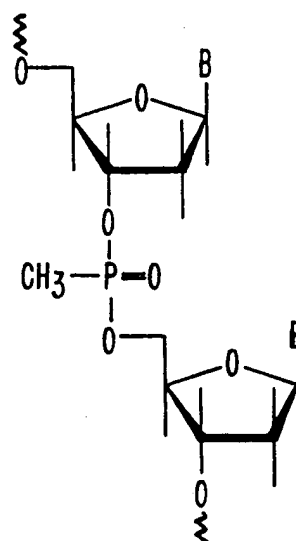
FIG. 7.



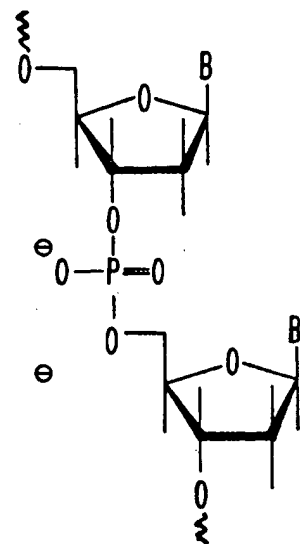
7/7



oligo-2'-O-methylribonucleoside  
methylphosphonate



oligodeoxyribonucleoside  
methylphosphonate



oligodeoxyribonucleoside

FIG. 8.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/00157

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 31/70; C07H 2102

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/044; 536/24.3, 24.3, 24.5, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| A         | US, A, 4,839,177 (COLOMBO ET AL.) 13 June 1989, entire document.   | 1-30                  |
| A         | J. AMER. CHEM. SOC., Volume 94, No. 13 issued 28 June 1972, Hevesi et al., "Contributions to the Mechanism of the Acid-Catalyzed Hydrolysis of Purine Nucleoside," pp. 4715-4720, entire document. | 1-30                  |
| A,P       | CHEMICAL & ENGINEERING NEWS, Vol. 71, No. 41, issued 11 October 1993, Nielsen et al., "Peptide' Nucleic Acid Hybridizes to Complementary Oligonucleotide," p. 25, entire document.                 | 1-30                  |

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

|   |     |  |
|---|-----|--|
| * Special categories of cited documents:  | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| "A" document defining the general state of the art which is not considered to be part of particular relevance   | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| "E" earlier document published on or after the international filing date  | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "g" | document member of the same patent family  |
| "O" document referring to an oral disclosure, use, exhibition or other means  |     |  |
| "P" document published prior to the international filing date but later than the priority date claimed  |     |  |

Date of the actual completion of the international search

25 FEBRUARY 1994

Date of mailing of the international search report

18 MAR 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

L. ERIC CRANE

Telephone No. (703) 308-0196

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

514/044; 536/24.3, 24.3, 24.5, 25.3